SUPPLEMENTAL MATERIAL

Supplemental Methods

Human samples

DNA methylation patterns from human femoral artery atherectomy samples (n=22) were compared to normal mammary artery samples (n=9). Atherectomy samples were collected at vascular atherectomy operations19. The nine normal samples represent trimmed ends of mammary arteries isolated during cardiac bypass surgery. Average age (±SD) of the patients for plaques and normal arteries were 70,9 ±7,3 and 67,2 ± 9,1 years, respectively (Table1). No significant differences were found in major cardiovascular risk factors between atherosclerotic and control samples. The studies were approved by Local Ethical Committee of Kuopio University Hospital under identification number 53/2011 and the subjects gave informed consent.

Sample preparation

One half of the atherectomy samples was snap frozen in liquid nitrogen and the second half was processed for paraffin embedding. Total RNA, DNA and protein were isolated from frozen samples by the Trizol method (Cat# 15596-018, Life technologies, Paisley, United Kingdom).Total RNA used for microarray analysis was repurified using RNeasy silica membrane columns (Cat# 74106; Qiagen AB, Sollentuna, Sweden). Total RNA integrity was checked on the Agilent 2100 (Agilent, Santa Clara, California, USA), and only samples with RNA integrity number over 5 were used for gene expression analysis.

Dissolved DNA samples were repurified using DNeasy columns (Cat# 69506, Qiagen AB, Sollentuna, Sweden). DNA concentration was measured on a Qubit 2.0 Fluorometer (Cat# Q32866, Invitrogen, Carlsbad, California, USA) with PicoGreen assay kit(Cat# P7589, Invitrogen) on 480/520 nm. RNA concentration was estimated on Nanodrop 2000 UV-VIS spectrophotometer (Thermo Scientific, Wilmington, Delaware, USA) spectrophotometer at 260 nm wavelength.

Protein samples from Trizol procedure were further purified as described [2] and after solubilzation in 4M urea, the protein concentration was estimated by bicinchoninic acid (BCA) protein assay (Cat# 23227, Thermo Scientific, Wilmington, Delaware, USA).

DNA methylation analysis

DNA samples were fragmented on Covaris S2 with following settings: duty cycle 10%, intensity 5, 200 cycles per burst during 190 sec to obtain fragments with an average length of 200 bp. The power mode was frequency sweeping, temperature 6 – 8^{o}C, water level 12. Maximum 3 µg was loaded in 130 µl TE in a microtube with AFA intensifier (Covaris, Woburn, Massachusetts, USA). Fragment distribution was checked on a high sensitivity chip on the Agilent 2100 (Agilent). Methylated DNA fragments were captured using the MethylCap kit (Diagenode AF-100-0048, Belgium). The concentrations of the fragmented and captured DNA were determined on a Fluostar Optima plate reader (BMG Labtech, Offenburg, Germany) with the PicoGreen dsDNA assay kit (P7589, Invitrogen) on 480/520 nm.

Library preparation for sequencing was a modification of the "multiplexed paired end ChIP protocol'' (Illumina, San Diego, California, USA). Sample Prep Master Mix Set 1 (NEB E6040) was used in combination with the Multiplexing Sample Preparation Oligo Kit (Illumina PE-400-1001). We used 250 ng of fragmented DNA and performed library preparation on Apollo 324 with PrepX-DNA Library Kit (IntegenX Cat# 400040).

Library amplification according to multiplexed paired end ChIP protocol included indexes from Multiplexing Preparation Oligo Kit. Smaller fragments were removed by 2% agarose gel electrophoresis (Low Range Ultra agarose BioRad 161-3107, BioRad, Hercules, California, USA). We used a 1Kb plus Ladder (Cat# 10787-018, Invitrogen) and gels were run for 2 h at 120V. Fragment range 300 ± 50 bp was excised and eluted on a Qiagen Gel Extraction kit column (Cat # 28704, Qiagen), eluted in 23 µl elution buffer and 1 µl was applied to Agilent 2100 HS DNA chip for smear analysis. Samples were diluted 1: 500, amplified by PCR and all samples were diluted to 10 nM. The paired end flow cell for Illumina HiSeq 2000 was prepared according to the Cluster Station User Guide manual (Illumina) and sequencing was performed for 2x51 cycles.

Paired end sequencing reads (51 bp) were mapped to human reference genome GRCH37/hg19 using Bowtie v0.12.9 software.[3] The Bowtie parameters were set to 0 mismatches in the seed (first 28 nucleotides). Only unique reads were retained and both fragments must be located within 400 bp of each other on the human reference genome GRCH37/hg19. Multiple paired reads with the exact same location in one sample were discarded, as these are most likely amplified from the same sequence. Methylated regions were annotated using Homo Sapiens Ensembl database v56 and binned to promoter (from -2000 to +500 from transcription start site), exon (with exon number), intron and intergenic compartments.

Statistical analysis for differential methylation was performed using HOMER package[4] (http://homer.salk.edu/homer/) on uniquely mapped reads from normal mammary artery and atherosclerotic femoral artery samples applying false discovery rate (FDR) <0.05. Genetic compartments demonstrating >2.5 fold differential in DNA methylation were subjected to gene ontology analysis.

Microarray analysis

Affymetrix HGU133 Plus 2 microarrays (Affymetrix, Santa Clara, California, USA) comprising 54675 probe sets were utilized essentially as recommended by the manufacturer exploiting 3' IVT Express Kit (Affymetrix) for probe preparation and GeneChip® Hybridization, Wash, and Stain Kit (Affymetrix) for hybridization and detection. The raw data was submitted to Genome Omnibus public repository (http://www.ncbi.nlm.nih.gov/geo/) and the analysis followed standard procedures including Robust Multichip Average (RMA) data normalization.[5] After RMA normalization of the microarray raw data, a filtering step was applied to remove the weakest signals (intensities lower than 2x above global background) and from initial 54675 probe sets 17 589 were included in Significance Analysis of Microarrays (SAM).[6] SAM yielded 1628 differentially expressed genes (False Discovery Rate <0.1).

Quantitative Real-time PCR (QRT-PCR)

QRT-PCR was performed in duplicates (15 µl per well including cDNA equal to 10 ng of total RNA) using SYBR Green chemistry (Applied BioSystems, Foster City, CA, USA) and the ABI StepOne Plus apparatus (Applied BioSystems, Foster City, CA, USA). The results were normalized to the geometrical mean of three genes (B2M, PPIA, RPLPO) showing the least variation from sample to

sample. The three normalizing genes were selected from a total of six housekeeping genes (ACTB, B2M, GUSB, PPIA, RPLPO, TUBB). The primers (table below) were designed using Primer3.[7]

Gene	Forward Primer	Reverse Primer
DLK1	5'-AGGAGATCTAAGCAGCGTTCC-3'	5'-CCCTCCCATTTAAGGTGGTTA-3'
DIO3	5'-ACTGGTCTTGACCACGAGCTA-3'	5'-CCCTCCCATTTAAGGTGGTTA-3'
RTL1	5'-GTCCTGGAATTCCTTGGTAGC-3'	5'-TCATCAGGCAGCTCTTCTAGC-3'
LNC00523	5'-CTCACTCGAGACCACTCCAAG-3'	5'-CTGGAGGATGGTTTTCTGACA-3'
MEG3	5'-CACAGAGTGGGGCTCAGTTAG-3'	5'-AAACCAGGAAGGAGACGAGAG-3'
MEG8	5'-AAGAAGACCAGCCTTCCAGAC-3'	5'-TTCGAGGCAGTTTTCACTGAG-3'
B2M	5'-TAGAGGTGGGGAGCAGAGAA-3'	5'-TCCCCCAAATTCTAAGCAGA-3'
PPIA	5'-TGGTGTTTGGCAAAGTGAAA-3'	5'-TCGAGTTGTCCACAGTCAGC-3'
RPLPO	5'-TCATCAACGGGTACAAACGA-3'	5'-GCCTTGACCTTTTCAGCAAG-3'

miRNA expression detection

Total RNA (20 ng) was taken for cDNA synthesis in volume of 20 µl using miRCURY LNA™ Universal RT kit (Cat #203300, Exiqon, Vedbaek, Denmark). After synthesis the cDNAs were diluted 80-fold and 4 µl was taken for QRT-PCR. Specific miRNAs (hsa-miR-127, -136, -410, -432, and -433) were detected with commercial SYBR Green QRT-PCR assays from Exiqon (Vedbaek, Denmark) on ABI StepOne Plus apparatus (Applied BioSystems, Foster City, CA, USA). The same three housekeeping genes (B2M, PPIA, RPLPO) were used as above.

Western blot

Solubilized protein samples (20 – 30 µg) in 4M urea (see Sample Preparation section) were applied to commercial gradient SDS gels (4 - 15%, Cat#456-1084; BioRad), electrophoresed for 1h at 160V. Blotting (semidry TransBlot SD apparatus; BioRad) onto 0.45µm nitrocellulose filter paper (Cat# 162-0115, BioRad)was performed at 25Vfor 45 min essentially as described.[8] Blots were blocked in 2.5% skimmed milk. Primary anti-RTL1 (rabbit polyclonal diluted 1:100, Cat# AB107990) was purchased from Abcam (Cambridge, United Kingdom) and used as suggested by the supplier. Goat anti-rabbit secondary antibody used in Western blotting was purchased from Thermo Scientific (Pierce Cat#31460; Thermo Scientific, Waltham, Massachusetts, USA). Blots were developed using Pierce ECL2 Western Blot substrate (Cat#32132, Thermo Scientific, Waltham, Massachusetts, USA) and digitized using Typhoon 9410 Molecular Imager (Amersham; Buckinghamshire, United Kingdom).

Immunohistochemistry and immunofluorescence

Paraffin-embedded human atherosclerotic lesions and mammary arteries were cut into thin (4 µm thick) sections, deparaffinized in xylene (2 x 10 min), rehydrated in graded alcohol series (100%, 95%, 70%, 50%, 5 min each), washed once with PBS (5 min) followed by 0.25% TritonX100 in PBS (10 min) and endogenous peroxidase inactivation (3% H2O2 in PBS, 20 min, done only in case of immunohistochemical antigen detection). Antigen retrieval was performed by boiling the samples (in microwave oven using 10 mM TRIS, 1 EDTA, pH9.0, 15 min and followed by cooling down to room temperature, 30 min). Slides were rinsed in PBS (5 min) and blocked against unspecific binding (2% human serum, 10% horse or goat serum, 1% BSA in PBS, 30 – 60 min at room temperature). Primary antibody (diluted in blocking solution) incubation was carried out over night at +4oC. On the next day, slides were washed with PBS (3 x 5 min) and incubated with secondary antibody in blocking solution for 30 – 60 min.

Secondary antibodies for immunohistochemistry were from Vector Laboratories (horse anti-mouse HRP-conjugated, 1 : 200, Cat# BA-2000 and horse anti-rabbit biotinylated; 1 : 200). Avidin-biotin-HRP (ABC reagent) was prepared 30 min before use (2 drops of A + B per 5 ml PBS, Vector Laboratories) and incubated for 30 min. PBS wash (3 x 5 min) was followed by DAB chromogen (1 drop of each A+B+C + 1 ml H2O; DAB plus substrate kit, Invitrogen Cat#062020), water wash (2 – 5 min), Counterstaining with Harris haematoxylin (20 seconds), wash in running tap water (2 – 5 min), dehydratation in graded alcohol series (50%, 70%, 95%, 100%; 2 min each), xylene wash and mounting in Permount (Fischer Cat#SP15-500).

Samples were incubated with the following secondary antibodies for immunofluorescence: goat anti-mouse ALEXA 488-labeled (1 : 200, Cat#A11029, Invitrogen) and goat anti-rabbit ALEXA 594-labeled (diluted 1 : 200, Cat#A11012, Invitrogen), and counterstained with HOECHST33342 (Invitrogen;for working solution dilution 1:1000 in PBS was prepared, 10 min), PBS washed (3x 5 min), mounted in Cytoseal60 and imaged immediately.

Primary antibodies were the same in both instances. SM Actin antibody was a mouse monoclone to human SMA (working dilution 1: 100, clone 1A4, Dako Cat# M0851). CD68 antibody was a mouse monoclonal to human protein (working dilution 1: 200; KP1; Santa Cruz Biotechnology Cat# sc-20060). RTL1 was a rabbit polyclonal (working concentration 1.100) from Abcam (Cat# ab107990).

Statistical and bioinformatical analysis

NGS reads were mapped to human chromosomes to predefined genetic compartments (promoters at-2000+500 from transcription start sites (TSS), exons and introns according to the genome update GRCh37 coordinates provided by the ENSEMBL Genome Browser (http://www.ensembl.org/index.html), and the number of unique reads per sample was approximately 6 - 14 million out of 30 – 35 million raw reads. The counts for uniquely mapped reads were normalized to represent a total of 10 million uniquely mapped reads per sample by adjusting the number of reads at each compartment in the genome to the correct fractional amount given the total reads mapped. After read count normalization statistically significant differences at specific loci were identified. Fold-change differences in DNA methylation were defined for each genomic compartment (atherosclerotic samples vs. normal) using locally installed HOMER software[4] (http://homer.salk.edu/homer/) at estimated false discovery rate FDR <0.05. Ingenuity Pathway Analysis package (Ingenuity, Redwood City, California, USA) was used for bioinformatical analyses of genes showing significantly ($p<0.05$) and prominently (cut off value was set to 2.5x fold difference) modified DNA methylation patterns.

Microarray analysis (RMA normalization followed by filtering to remove weak signals and definition of differential expression) was performed using Chipster program package.[9]

The rest of the statistical analyses were performed using SPSS14 for Windows (SPSS Inc., Chicago, Illinois, USA) and GraphPad Prism (GraphPad, La Jolla, CA, USA). Independent-samples T-test was used for parametric variables and Mann-Whitney U-test for non-parametric variables. Correlations between parametric variables were determined by Pearson's algorithm. Categorical patient data was analysed using Fisher Exact Test.

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